

## **Supplemental *Salmonella typhimurium* mutagenicity tests with 4 anthraquinone samples**

### **A. Identity of the 4 samples tested**

1. A07496. 9,10-Anthraquinone, Lot #5893, from Zeneca Fine Chemicals, generated using the Nitric Acid Oxidation manufacturing process. This is the sample from the NTP 2-year bioassay. An aliquot of this sample was also tested by Butterworth et al. (2001) in the *Salmonella* mutagenicity test.
2. A65343. 9,10-Anthraquinone, Lot #64005, obtained from Environmental Biocontrol Intl., generated using the Diels-Alder manufacturing process.
3. A54984. 9,10-Anthraquinone, Lot # GSTU 2517770, obtained from Environmental Biocontrol Intl., generated using the Friedel-Crafts manufacturing process.
4. A40147. 9,10-Anthraquinone, Lot #2Y011, obtained from Kawasaki Kasei Chemical, LTD, generated using the Diels-Alder manufacturing process.

### **B. *Salmonella* test protocol**

Testing was performed as reported by Zeiger et al. (1992), with modifications as described below. The test aliquots (selected to represent a broad sampling of anthraquinones produced by different manufacturing processes) were sent to the testing laboratory as coded aliquots. They were incubated with the *Salmonella typhimurium* tester strains either in buffer or S9 mix (metabolic activation enzymes and cofactors from Aroclor 1254-induced male Sprague Dawley rat liver) for 20 minutes at 37 ° C. All 4 samples were tested in *Salmonella typhimurium* strains TA98 and TA100; the bioassay sample, #A07496, was also tested in strain TA1537. The NTP added this third strain in the test of the bioassay sample in order to independently assess the mutagenicity of this particular anthraquinone sample in the tester strains that gave positive results in the studies reported by Butterworth et al. (2001).

After the 20-minute preincubation period, top agar supplemented with L-histidine and d-biotin was added, and the contents of the tubes were mixed and poured onto the surfaces of minimal glucose agar plates. Histidine-independent mutant colonies arising on these plates were counted following two days incubation at 37° C.

Each trial consisted of triplicate plates of concurrent positive and negative controls and of at least 5 doses of the anthraquinone aliquot. A number of considerations impacted the selection of test article concentrations. Butterworth et al. (2001) tested the NTP bioassay anthraquinone sample A07496 over a range of 30 – 2000 ug/plate; the sample originally tested for mutagenicity by the NTP (97% pure; different from the bioassay material) was tested over a range of 33-2500 ug/plate, with toxicity being the dose-limiting factor in this test. The purified NTP sample, reported in the technical report that was reviewed 2/18/04, was tested over a concentration range of 100 – 10,000 ug/plate. Thus, to cover

all dose ranges, test article concentrations for this supplemental testing effort were designated by the NTP to be: 30, 100, 300, 1000, 3000, and 10,000 ug/plate, with and without 10% rat liver S9 enzymes. In the case of the bioassay sample, A07496, testing was also conducted with 30% rat liver S9, to match the protocol that the NTP used in testing the original anthraquinone aliquot (97% pure) (Zeiger et al., 1988).

### **Positive control agents**

In the absence of metabolic activation, the positive controls were sodium azide (TA100), 9-aminoacridine (TA1537), and 4-nitro-o-phenylenediamine (TA98). The positive control for all strains tested with metabolic activation was 2-aminoanthracene.

### **The selection of solvent in these special supplemental tests**

Dimethylsulfoxide (DMSO) was the solvent used in the first NTP test of anthraquinone (97% pure) (Zeiger et al., 1988). The second, more recent test of a different sample of anthraquinone (100% pure) used propylene glycol as the solvent. Butterworth et al. (2001) did not specify the solvent used in their *Salmonella* studies with anthraquinone. In an approach designed to be comprehensive, it was decided to test the bioassay sample of anthraquinone in both solvents used previously in NTP tests (DMSO and propylene glycol). DMSO was used for the other 3 anthraquinone samples listed above.

### **Data evaluation**

In the *Salmonella* mutagenicity assay, a positive response is defined as a reproducible, dose-related increase in histidine-independent (revertant) colonies in any one strain/activation combination. An equivocal response is defined as an increase in revertants that is not dose-related, is not reproducible, or is not of sufficient magnitude to support a determination of mutagenicity. A negative response is obtained when no increase in revertant colonies is observed following chemical treatment. There is no minimum percentage or fold-increase required for a chemical to be judged positive.

## Results

The *Salmonella* mutagenicity test results for the NTP bioassay sample of anthraquinone (A07496) were negative in all three tester strains, with and without 10% or 30% rat liver S9, at concentrations up to 10,000 ug/plate, with both solvents (Table 1).

Samples A65343 (Table 2) and A54984 (Table 3) were negative in TA98 and TA100, with and without S9, at concentrations up to 10,000 ug/plate. These samples were not tested in TA1537.

Sample A40147, produced by the Diels-Alder manufacturing process and obtained by the NTP from Kawasaki Kasei Chemical, LTD, was mutagenic in *Salmonella typhimurium* strains TA100 and TA98, with and without S9 (Table 4). The lowest effective dose of anthraquinone in this test was 100 ug/plate in TA98 without S9, and 1000 ug/plate in TA98 with S9. The response in TA100 was less impressive, with the lowest effective dose being approximately 3000 ug/plate with S9 and 10,000 ug/plate in the absence of S9 (this was the highest dose tested, and is higher than most screening laboratories use routinely in the absence of dose-limiting toxicity).

### 1. Mutagenicity of Anthraquinone (A07496) in *Salmonella typhimurium*

#### TA100

Solvent: S9: Result:	PG NA Negative	DMSO NA Negative	PG 10% Negative	DMSO 10% Negative	PG 30% Negative	DMSO 30% Negative
ug/Plate	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
0	97 ± 9.2	97 ± 6.4	139 ± 4.8	112 ± 3.5	114 ± 6.1	116 ± 10.0
30	84 ± 2.3	84 ± 13.8	123 ± 11.0	103 ± 6.0	127 ± 3.1	118 ± 5.4
100	86 ± 4.6	93p ± 9.3	97 ± 2.3	118p ± 2.9	132 ± 2.3	100 ± 7.6
300	82 ± 4.6	101p ± 3.2	108 ± 0.9	117p ± 8.4	134 ± 2.6	99p ± 1.2
1000	88p ± 6.3	103p ± 2.9	99p ± 6.4	113p ± 6.7	126p ± 1.5	64p ± 24.0
3000	70p ± 7.8	88p ± 1.5	110p ± 1.8	120p ± 0.9	125p ± 8.8	105p ± 6.2
10000	78p ± 11.9	85p ± 6.1	124p ± 4.4	125p ± 7.9	129p ± 1.2	109p ± 4.6
Pos. control	512 ± 13.0	436 ± 38.6	514 ± 6.7	534 ± 37.7	414 ± 10.6	486 ± 38.0

# 1. Mutagenicity of Anthraquinone (A07496) in *Salmonella typhimurium* (cont'd)

## TA1537

Solvent: S9: Result:	PG NA Negative	DMSO NA Negative	PG 10% Negative	DMSO 10% Negative	PG 30% Negative	DMSO 30% Negative
ug/Plate	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
0	7 ± 2.2	7 ± 0.6	7 ± 2.1	10 ± 0.6	11 ± 0.6	10 ± 1.0
30	5 ± 0.6	6 ± 0.3	5 ± 0.6	7 ± 0.7	10 ± 1.2	8 ± 2.5
100	4 ± 0.6	4p ± 1.2	7 ± 1.9	10p ± 1.7	8 ± 2.3	9p ± 0.9
300	6 ± 1.0	5p ± 0.9	5 ± 1.2	6p ± 1.8	6 ± 0.7	10p ± 1.2
1000	7p ± 2.2	4p ± 1.5	6p ± 2.2	10p ± 0.9	11p ± 2.3	13p ± 0.3
3000	6p ± 1.5	6p ± 1.2	8p ± 2.0	8p ± 1.2	11p ± 0.6	12p ± 0.9
10000	5p ± 2.0	6p ± 1.0	7p ± 1.5	11p ± 0.0	12p ± 0.6	17p ± 1.8
Pos. control	249 ± 36.4	90 ± 5.51	198 ± 10.3	230 ± 24.7	57 ± 7.3	72 ± 1.7

## TA98

Solvent: S9: Result:	PG NA Negative	DMSO NA Negative	PG 10% Negative	DMSO 10% Negative	PG 30% Negative	DMSO 30% Negative
ug/Plate	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
0	13 ± 1.7	16 ± 3.8	26 ± 3.2	31 ± 2.2	34 ± 3.5	28 ± 0.3
30	10 ± 1.9	13 ± 1.2	22 ± 3.2	17 ± 1.7	24 ± 2.6	25 ± 2.0
100	11 ± 0.9	13 ± 1.5	28 ± 0.0	25 ± 2.6	25 ± 2.7	21p ± 0.9
300	17 ± 1.2	17p ± 2.0	24 ± 3.0	33 ± 3.2	24 ± 0.7	28p ± 4.5
1000	14p ± 0.9	12p ± 1.2	28p ± 2.6	30p ± 6.6	25p ± 2.5	27p ± 3.5
3000	16p ± 1.2	14p ± 0.0	22p ± 2.3	24p ± 2.5	28p ± 0.3	33p ± 3.1
10000	14p ± 2.4	13p ± 4.9	32p ± 2.0	38p ± 2.6	35p ± 4.0	32p ± 3.4
Pos. control	56 ± 19.0	83 ± 7.2	295 ± 38.7	271 ± 29.0	190 ± 5.0	180 ± 24.3

p = Precipitate

DMSO = dimethylsulfoxide

PG = propylene glycol

Note: This test of the NTP bioassay sample was conducted in 3 strains of *Salmonella* (TA100, TA1537, TA98), using the preincubation protocol and 2 different solvents – propylene glycol and DMSO. The NTP also used 2 concentrations of Aroclor 1254-induced rat liver S9. These variations were included to repeat testing conditions described by Butterworth et al. (2001) and to include testing parameters investigated in previous studies by the NTP.

## 2. Mutagenicity of Anthraquinone (A65343) in *Salmonella typhimurium*

### TA100

S9: Result:	NA Negative	10% Negative
ug/Plate	Mean $\pm$ SE	Mean $\pm$ SE
0	113 $\pm$ 7.9	144 $\pm$ 1.5
30	115 $\pm$ 12.4	127 $\pm$ 6.7
100	113 $\pm$ 11.7	133 $\pm$ 5.8
300	114 $\pm$ 2.3	124 $\pm$ 9.4
1000	108p $\pm$ 7.1	133p $\pm$ 3.8
3000	130p $\pm$ 2.9	127p $\pm$ 9.2
10000	120x $\pm$ 2.1	132x $\pm$ 5.2
Pos. control	564 $\pm$ 7.1	519 $\pm$ 33.1

### TA98

S9: Result:	NA Negative	10% Negative
ug/Plate	Mean $\pm$ SE	Mean $\pm$ SE
0	16 $\pm$ 0.3	33 $\pm$ 2.4
30	13 $\pm$ 0.3	29 $\pm$ 1.2
100	15 $\pm$ 2.5	25 $\pm$ 1.0
300	18 $\pm$ 1.3	26 $\pm$ 1.5
1000	17p $\pm$ 1.7	30p $\pm$ 4.4
3000	17p $\pm$ 2.0	31p $\pm$ 2.3
10000	17x $\pm$ 2.3	31x $\pm$ 3.0
Pos. control	105 $\pm$ 3.7	400 $\pm$ 29.5

p = Precipitate; x = Slight Toxicity and Precipitate

### 3. Mutagenicity of Anthraquinone (A54984) in *Salmonella typhimurium*

#### TA100

<b>S9: Result:</b>	<b>NA Negative</b>	<b>10% Negative</b>
ug/Plate	Mean $\pm$ SE	Mean $\pm$ SE
0	132 $\pm$ 0.9	136 $\pm$ 5.2
30	120 $\pm$ 6.1	122 $\pm$ 11.3
100	140 $\pm$ 15.3	147 $\pm$ 5.0
300	138 $\pm$ 4.6	133 $\pm$ 7.0
1000	138p $\pm$ 13.2	138p $\pm$ 7.8
3000	129p $\pm$ 5.2	140p $\pm$ 2.7
10000	143p $\pm$ 13.0	127p $\pm$ 7.5
Pos. control	581 $\pm$ 38.5	522 $\pm$ 25.5

#### TA98

<b>S9: Result:</b>	<b>NA Negative</b>	<b>10% Negative</b>
ug/Plate	Mean $\pm$ SE	Mean $\pm$ SE
0	20 $\pm$ 0.9	24 $\pm$ 3.1
30	18 $\pm$ 1.5	33 $\pm$ 3.8
100	22 $\pm$ 4.1	27 $\pm$ 3.2
300	17 $\pm$ 0.7	29 $\pm$ 3.5
1000	18p $\pm$ 1.2	33p $\pm$ 2.6
3000	20p $\pm$ 0.9	29p $\pm$ 0.9
10000	17p $\pm$ 1.2	31p $\pm$ 1.7
Pos. control	105 $\pm$ 9.7	415 $\pm$ 1.2

p = Precipitate

#### 4. Mutagenicity of Anthraquinone (A40147) in *Salmonella typhimurium*

##### TA100

<b>S9: Result:</b>	<b>NA Weak Positive</b>	<b>10% Positive</b>
ug/Plate	Mean $\pm$ SE	Mean $\pm$ SE
0	154 $\pm$ 10.0	108 $\pm$ 7.5
30	136 $\pm$ 5.5	108 $\pm$ 7.5
100	141 $\pm$ 1.5	133 $\pm$ 6.2
300	143p $\pm$ 2.9	133p $\pm$ 5.7
1000	77p $\pm$ 4.3	140p $\pm$ 6.2
3000	154p $\pm$ 6.1	182p $\pm$ 3.9*
10000	531p $\pm$ 33.1*	389p $\pm$ 23.1*
Pos. control	535 $\pm$ 8.5	530 $\pm$ 30.9

##### TA98

<b>S9: Result:</b>	<b>NA Positive</b>	<b>10% Positive</b>
ug/Plate	Mean $\pm$ SE	Mean $\pm$ SE
0	13 $\pm$ 2.3	32 $\pm$ 2.3
30	21 $\pm$ 2.2	31 $\pm$ 4.4
100	36 $\pm$ 1.2*	42 $\pm$ 4.7
300	45p $\pm$ 1.9*	41p $\pm$ 4.4
1000	108p $\pm$ 7.2*	72p $\pm$ 9.8*
3000	279p $\pm$ 14.4*	174p $\pm$ 29.4*
10000	932p $\pm$ 72.2*	731p $\pm$ 16.0*
Pos. control	95 $\pm$ 1.5	491 $\pm$ 22.7

p = Precipitate

\*significant increases in revertant colonies